REMARKS

Claims 2-6, 8, 23-40, and 42 are pending in the application. Claims 23-40 and 42 are withdrawn as being drawn to non-elected inventions. Claims 1, 7, and 41 are canceled without prejudice or disclaimer. Claims 2-6 and 8 are under active consideration.

In order to expedite prosecution, claim 23 has been amended and claims 1, 7, and 41 have been canceled without prejudice or disclaimer in order to remove non-elected subject matter. Applicants reserve the right to prosecute non-elected subject matter in subsequent divisional applications.

Cancellation and amendment of the claims is made without prejudice, without intent to abandon any originally claimed subject matter, and without intent to acquiesce in any rejection of record. Applicants expressly reserve the right to file one or more continuing applications hereof containing the canceled or unamended claims.

Restriction Requirement

Applicants affirm the election without traverse of Group II, which corresponds to claims 2-6 and 8, directed to a fusion protein and a composition consisting essentially of the fusion peptide or isolated HCV polypeptides, wherein the fusion protein and isolated polypeptides consist essentially of NS3, NS4, NS5a, NS5b and core.

Rejoinder

Applicants request that claims 23-33, 37-40, and 42, drawn to methods of using the fusion proteins and compositions of Group II, be rejoined per the Commissioner's Notice in the Official Gazette of March 26, 1996, entitled "Guidance on Treatment of Product and Process Claims in light of In re Ochiai, In re Brouwer and 35 U.S.C. § 103(b)" which sets forth the rules, upon allowance of product claims, for rejoinder of process claims covering the same scope of products. Applicants request that claims 23-33, 37-40, and 42 be rejoined and examined upon allowance of any of the claims drawn to the fusion proteins of Groups II.

Priority

The Office Action states that the subject matter of claims 2-6 and 8 is not entitled to the benefit of priority of the provisional application 60/161,713, filed October 27, 1999 because the provisional application allegedly "fails to provide adequate support under 35 U.S.C. 112 for claims 2-6 and 8 of this application" (Office Action, page 2). The Office Action further alleges that the subject matter of claims 2-6 and 8 is not entitled to the benefit of priority of U.S. Application Serial No. 09/698,874, filed October 27, 2000 and U.S. Application Serial No. 10/357,619, filed February 3, 2003; therefore the claimed priority back to the filing dates of October 27, 2000 and February 3, 2003 have also been denied (Office Action, page 2).

Applicants respectfully disagree. U.S. Application Serial No. 09/698,874 and U.S. Application Serial No. 10/357,619 describe HCV fusion proteins comprising NS3, NS4a, NS4b, NS5a, NS5b, and a polypeptide from the core region. See the specifications, for example, at page 18, line 23 through page 19, line 6. Both applications also describe fusions comprising polypeptides from different HCV strains, for example, at page 18, lines 9-22; and isolated and purified polypeptides, for example, at page 12, lines 12-22; and page 16, lines 26-29. Therefore, the claims are entitled to the priority dates of October 27, 2000 and February 3, 2003. Nevertheless, Applicants do not need to rely on the earlier priority dates to overcome the cited art.

Written Description Rejection under 35 U.S.C. § 112, first paragraph

Claims 2-6 and 8 have been rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of an adequate written description. In particular, the Office Action alleges that applicants did not have possession of the claimed HCV fusion protein consisting essentially of an NS3, NS4, NS5a, NS5b and a core polypeptide of HCV as the application was originally filed (Office Action, page 3). The Office Action further alleges that "the specification does not teach a fusion protein or composition comprising the fusion protein that contains HCV core antigen in addition to NS3, NS4, NS5a and/or NS5b" (Office Action, page 4). Applicants respectfully traverse the rejection on the following grounds.

The fundamental factual inquiry in written description is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought,

applicant was in possession of the invention as now claimed. *See, e.g., Vas-Cath, Inc.*, 935 F.2d at 1563-64, 19 USPQ2d at 1117. Determining whether the written description requirement is satisfied is a question of fact and the burden is on the Examiner to provide evidence as to why a skilled artisan would not have recognized that the applicant was in possession of claimed invention at the time of filing. *Vas-Cath, Inc. v. Mahurkar*, 19 USPQ2d 1111 (Fed. Cir. 1991); *In re Wertheim,* 191 USPQ 90 (CCPA 1976). It is not necessary that the application describe the claimed invention *in ipsis verba.* Rather, all that is required is that the specification reasonably convey possession of the invention. *See, e.g., In re Lukach,* 169 USPQ 795, 796 (CCPA 1971). Finally, determining whether the written description requirement is satisfied requires reading the disclosure in light of the knowledge possessed by the skilled artisan at the time of filing, for example as established by reference to patents and publications available to the public prior to the filing date of the application. *See, e.g., In re Lange,* 209 USPQ 288 (CCPA 1981).

The specification as filed fully describes the claimed subject matter. Fusion proteins comprising HCV core antigens are described in the specification, for example, at page 18, line 24 through page 19, line 6. The specification explicitly states that "[i]n addition to NS3, NS4a, NS5a and NS5b, the fusion proteins can contain other polypeptides derived from the HCV polyprotein. For example, it may be desirable to include polypeptides derived from the core region of the HCV polyprotein" (page 18, lines 23-25). The specification further defines the core region as encompassing amino acid positions 1-191 of the HCV polyprotein and describes fusions that contain the full length core region or epitopes of the core region (see specification, for example, at page 18, lines 25-27). Therefore, fusion proteins comprising NS3, NS4a, NS5a, NS5b and core antigens are adequately described.

For at least the above reasons, withdrawal of the written description rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Double Patenting Rejections under 35 U.S.C. § 101

A. Statutory Double Patenting

Claims 2-6 and 8 have been provisionally rejected under 35 U.S.C. § 101 for statutory double patenting on the grounds that claims 3-6 and 10-11 of copending U.S. Application Serial

No. 10,281,341 allegedly claim the same invention. Claims in copending U.S. Application Serial No. 10,281,341 are currently being amended such that the claims no longer recite the same invention.

B. Nonstatutory Double Patenting

Claim 2 has been provisionally rejected under the judicicially created doctrine of obviousness-type double patenting as being unpatentable over claims 41-44 of copending U.S. Patent Application Serial No. 10,612,884. Applicants request that the requirement for submission of a Terminal Disclaimer with respect to U.S. Patent Application No. 10,612,884 be held in abeyance until there is an indication of allowable subject matter in the present application.

Rejections under 35 U.S.C. § 102

Claim 2 has been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by the reference of Grakoui et al. (1996) J. Virology 67:1385-1395. In particular, the Office Action alleges:

> Grakoui et al. teach a fusion protein comprising HCV polyprotein core, NS3, NS4, NS5a and NS5b, which is produced by the recombinant DNA construct pBRTM/HCV 1-2940, pBRTM/HCV 1-2813 or pBRTM/HCV 1-2508 (See Fig. 1 and Table 2).
> (Office Action, page 7.)

In addition, claim 2 has been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by the reference of Selby et al. (1993) J. Gene. Virol. 74:1103-1113. In particular, the Office Action alleges:

> Selby et al. teach a fusion protein comprising HCV polyprotein core, NS3, NS4, NS5a and NS5b, which is produced by the recombinant DNA construct pEMCV-HCV, or pHCVss or pβglo-HCV (See Fig. 1). Selby et al. also teach that the HCV fusion protein can be used to produce an antiserum comprising the antibodies against each domain of HCV polyprotein (See section of antiserum on page 1105).
> (Office Action, page 7.)

Claim 2 has also been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by the reference of Cheng et al. (1996) Clinical and Diagnostic Virology 6:137-145. In particular, the Office Action alleges:

Cheng et al. teach a fusion protein comprising HCV polyprotein core, NS3, NS4, NS5 (See section of Results). (Office Action, page 7.)

Applicants respectfully traverse the rejections under 35 U.S.C. § 102(b) on the following grounds.

For a reference to anticipate claimed subject matter under 35 U.S.C. § 102, "the reference must teach every aspect of the claimed invention either explicitly or implicitly." M.P.E.P. § 706.02. Applicants respectfully submit that the references of Grakoui et al., Selby et al., and Cheng et al. do not teach or suggest all aspects of the Applicants' invention, either explicitly or implicitly.

The reference of Grakoui et al. does not disclose a fusion protein consisting essentially of an NS3, an NS4, an NS5a, and a core polypeptide of a hepatitis C virus (HCV), as claimed. Grakoui fails to disclose an HCV fusion protein comprising an NS3 polypeptide in combination with a core domain or portion thereof that lacks the intervening regions (*i.e.*, E1, E2, p7, NS2), naturally occurring in the HCV polyprotein. Nor does Grakoui disclose fusion proteins, wherein one or more of the HCV polypeptides is derived from a different strain of HCV than the other HCV polypeptides. Therefore, Grakoui fails to teach all the limitations of the claims.

The reference of Selby et al. also fails to disclose any fusion protein containing core that lacks E1, E2, and NS2 (see Fig. 1 at page 1104). Therefore, the claims are not anticipated by Selby et al.

The reference of Cheng et al. fails to disclose a fusion protein comprising an NS5b polypeptide as claimed. Rather, Cheng et al. describe a fusion protein containing a single NS5 antigenic determinant consisting of residues 1-73 of the amino acid sequence disclosed by Maeno et al. (1990) Nucleic Acids Res. 18:2685-2688 (see Cheng et al. at page 141, col.2). The Examiner's attention is directed to the enclosed reference of Maeno et al. attached at Appendix A. The Maeno et al. sequence consists of a fragment of NS5a and contains no portion of NS5b. Therefore, the reference of Cheng et al. fails to teach all the limitations of the claims.

For at least these reasons, withdrawal of the rejections under 35 U.S.C. § 102(b) is respectfully requested.

Rejection under 35 U.S.C. § 103

Claims 2 and 8 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over the references of Selby et al. (1993) J. Gene. Virol. 74:1103-1113 and Alvarez-Lajonchere et al. (2002) Mern Inst. Oswaldo Cruz. 97:95-99. In particular, the Office Action alleges:

> [I]t would have been obvious to one of ordinary skill in the art at the time of the invention was filed to be motivated by the recited references and to combine the method taught by Selby et al. to prepare the HCV fusion protein and select one kind of adjuvant disclosed by Alvarez-Lajonchere et al. to make an immunogenic composition et al. in order to produce an enhanced immune response absence unexpected result.
> (Office Action, page 8).

Applicants respectfully traverse the rejection under 35 U.S.C. § 103 on the following grounds.

To support an obviousness rejection under 35 U.S.C. § 103, "all the claim limitations must be taught or suggested by the prior art." M.P.E.P. § 2143.03. In addition, "the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure." M.P.E.P. § 706.02.

Applicants submit that the cited references do not disclose or suggest all the limitations of the present invention. Thus, a *prima facie* case of obviousness has not been presented by the Office, and the cited combination is based on impermissible hindsight reconstruction.

As mentioned above, Selby et al. fails to disclose or suggest any fusion protein comprising core in the absence of E1, E2, and NS2. The reference of Alvarez-Lajonchere et al. also fails to teach or suggest such fusion proteins. Alvarez-Lajonchere et al. et al. (5,371,017) only describe the use of core antigens for generating an immune response; however, fail to describe or suggest any HCV fusion protein comprising core and other HCV sequences.

Thus, the references do not disclose or suggest all the limitations of the present invention, and the Examiner has not met the burden of establishing a *prima facie* case of obviousness. In the absence of some teaching or suggestion in the cited references concerning immunogenic fusion proteins consisting essentially of NS3, NS4, NS5a, NS5b, and core polypeptides, as described in the present application, the Examiner has presented no more than an improper hindsight reconstruction of the present invention. As stated by the Court of Appeals for the

Federal Circuit *In re Fine*, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988): "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention." Therefore, the Office has not met the requirements for a *prima facie* showing of obviousness under 35 U.S.C. § 103. For at least the above reasons, withdrawal of the rejections under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

In light of the above remarks, Applicants submit that the present application is fully in condition for allowance. Early notice to that effect is earnestly solicited.

If the Examiner contemplates other action, or if a telephone conference would expedite allowance of the claims, Applicants invite the Examiner to contact the undersigned.

The Commissioner is hereby authorized to charge any fees and credit any overpayment of fees which may be required under 37 C.F.R. §1.16, §1.17, or §1.21, to Deposit Account No. 18-1648.

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Enclosures:
1. Maeno et al. (1990) Nucleic Acids Res. 18:2685-2688.

A cDNA clone closely associated with non-A, non-B hepatitis

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ABSTRACT

A lambda gt11 cDNA library was constructed from RNA purified from hepatitis B viral surface antigen-negative human plasma with high alanine aminotransferase activity. A cDNA clone, designated as C8-2, was isolated by immunoscreening with mixed sera from non-A, non-B hepatitis (NANBH) carrier and convalescent chimpanzees. The recombinant protein produced by C8-2 reacted specifically with sera of patients in the chronic phase of NANBH. The sequence of C8-2, 269 bp, did not hybridized with any human or chimpanzee genomic DNA, and had no homology with those of primates and viruses. The existence of this sequence in RNA of possibly infectious plasma was shown by RNA blot hybridization and by Southern blot analysis of products amplified by the polymerase chain reaction. These results strongly suggest that C8-2 is derived from the agent of this viral hepatitis.

INTRODUCTION

Post-transfusion non-A, non-B hepatitis (NANBH) is a major clinical problems because it is thought to be the cause of up to 90% of all cases of post-transfusion hepatitis and of at least 50% of those of sporadic hepatitis (1). The transmissible agent of this hepatitis has not yet been identified, in spite of intensive studies using various conventional techniques (2–4). Thus the recent study by Choo and collaborators (5) was highly significant in that they isolated cDNA derived from viral RNA from plasma of an infected chimpanzee. They have also shown by enzyme-linked immunosorbent assay that antibody to this recombinant antigen (C100-3) appears specifically in the sera of patients with NANBH (6). These workers named this RNA virus hepatitis C virus (HCV).

Previously we constructed a cDNA library from pooled plasma of Japanese blood donors that possibly contained the infectious agent, and screened it using pooled sera from chronic carrier and convalescent chimpanzees that had been infected with the F-strain of the NANBH agent (7). In the present study, a clone of exogenous origin was isolated and shown to be derived from RNA in infectious human plasma. The antibody to the fusion protein expressed in *E. coli* was shown to be detected in patients in the chronic phase of NANBH.

MATERIALS AND METHODS

Construction of a lambda gt11 cDNA library from RNA of human plasma

Eight liters of hepatitis B viral surface antigen-free plasma from 148 Japanese blood donors whose alanine aminotransferase (ALT) values were more than 100 mIU/ml, were pooled. The viral fraction was precipitated with 3.6% (W/W) polyethylene glycol 6000 (Wako chem., Tokyo) and 500 mM NaCl, and a suspension of the precipitate was centrifuged on a stepwise sucrose gradient (5, 10, 15, and 20% (W/W)) for 12 hrs at 80000G. The resultant pellet was suspended with 8 ml of PBS (10 mM sodium phosphate buffer pH7.4, 150 mM NaCl).

The RNA was purified by the method of Chirgwin *et al.* (8) with slight modification. Five volumes of extraction buffer (4M guanidium thiocyanate, 50 mM Tris-HCl pH7.6, 10 mM ethylene diaminetetraacetic acid (EDTA), 100 mM 2-mercaptoethanol, 2% sarcosil) was added to the concentrated plasma described above, and total nucleic acid was extracted with phenol-chloroform, and precipitated with ethanol with 20 μg/ml glycogen as carrier. The RNA was further purified by DNase treatment (1.15KU/ml DNase, 50 mM Tris-HCl pH7.4, 1 mM EDTA, 10 mM $MgCl_2$) for 30 min at 37°C, and anion exchange chromatography (Qiagen pack-100, Diagen). Complementary DNA was synthesized with random primers, and cloned into lambda gt11, using a lambda gt11 cloning kit (Amersham).

Immunoscreening and immunoplaque assay

A library containing 1.2×10^6 plaque forming units was immunoscreened with 10-fold diluted mixed sera from 4 chimpanzees in the convalescent phase and one chimpanzee in the carrier phase of NANBH. All these chimpanzees had been

* To whom correspondence should be addressed

inoculated with the F-strain of NANBH agent as described elsewhere (9). For confirmation of the specificity of the immunoreaction of sera against the recombinant antigen with respect to NANBH diagnosis, a phage lysate from the candidate clone was mixed in a 1:1 ratio with control phage without insertion, plated on the same agar plate, and immunoassayed with sera from normal and NANBH virus-infected chimpanzees, and from a human panel of patients (see Fig. 1).

Western blot analysis

Y1089 was infected with lambda gt11 phage containing the C8-2 insert, and lysogens were induced as described by Snyder *et al*. (10). A fusion protein was expressed in Y1089 by addition of isopropyl beta-D-thiogalactopyranoside (IPTG), and pelleted cells were solubilized with RIPA buffer (100 mM Tris-HCl pH7.5, 1% sodium deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 300 mM NaCl). The lysate was separated in 7.5% SDS-polyacrylamide gel, and transferred to nitrocellulose paper. After treatment with 5% skim milk, the paper was incubated with 20-fold diluted patient's serum. The reactive material was detected with peroxidase-conjugated anti-human IgG and 4-chloro-1-naphthol (Sigma) as substrate. For determination of molecular weight of the C8-2/lacZ fusion protein, thyroglobulin (330kd), ferritin (220kd), phosphorylase b (94kd) and albumin (67kd) were used as standard proteins to establish calibration curve.

Sequence analysis

The cDNA inserts obtained by immunoscreening were subcloned into the *EcoRI* site of pUC118 (Takara, Tokyo) and both strands of DNA were sequenced by the dideoxy nucleotide chain termination technique (11). Sequencing and protein analysis were performed by the programs supplied by DNASIS software (Hitachi, Tokyo).

Southern blot analysis and RNA dot hybridization

Human and chimpanzee genomic DNA, purified from leukocytes and liver, respectively, were digested overnight with *EcoRI* at 37°C, subjected to electrophoresis on 1% agarose gel, and transferred to a nitrocellulose membrane. The blot was hybridized for 16 hr at 42°C with ^{32}P-labeled C8-2-5 DNA probe excised from plasmid in a mixture containing 50% formamide, 5× Denhardt's solution, and 5× SSC (150 mM NaCl, 15 mM trisodium citrate) and then washed 4 times with 0.1× SSC containing 0.2% SDS at 68°C. The beta-actin gene with 1.5 kb purified probe was used as a positive control.

For detection of RNA in serum, total nucleic acid was isolated from 50 ml of pooled human plasma with high ALT activity by the same method as used for preparation of the cDNA library. Precipitated nucleic acid was resuspended in 40 μl of FA/FA buffer (6.5% formaldehyde, 50% formamide, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 0.5 mM EDTA pH 8.0), spotted on a nitrocellulose paper, and hybridized with C8-2-5 DNA probe. Treatment with RNase (30 μg/ml) was performed in buffer containing 50 mM Tris-HCl (pH. 7.4), 1 mM EDTA, and 10 mM $MgCl_2$.

Enzymatic amplification of complementary DNA by the polymerase chain reaction

The polymerase chain reaction (PCR) from RNA template was performed as described by Gama *et al.* (12). RNA from either the serum or liver was reverse-transcribed at 37°C for 90 min in the presence of 4 KU/ml reverse transcriptase (BRL), 50mM Tris-HCl pH 8.3, 6mM $MgCl_2$, 40mM KCl, 1mM dithiothreitol, 1mM dNTPs, 1.3KU/ml RNasin, and 30mg/ml random primers. Part of the reaction mixture was then added to 50 μl of amplification mixture containing 20 U/ml *Thermus aquaticus* polymerase and 250 nM of sense (5′-CCGACCCCTCCCACATTACAGCA-3′) and antisense (3′-CGTCTGGGGAGTCATGACGGGT-5′) primers. Forty amplification cycles, consisting of denaturation at 94°C (30 sec), annealing at 55°C (30 sec), and extension at 72°C (60 sec) were carried out in a DNA Thermal Cycler (Perkin-Elmer/Cetus). Amplified DNA was separated electrophoretically in 2% agarose gel, and Southern blot analysis was performed under usual stringent condition using synthetic oligo nucleotide (5′-TAACTGACTAGCTGAAGAGCTGGCCA-3′).

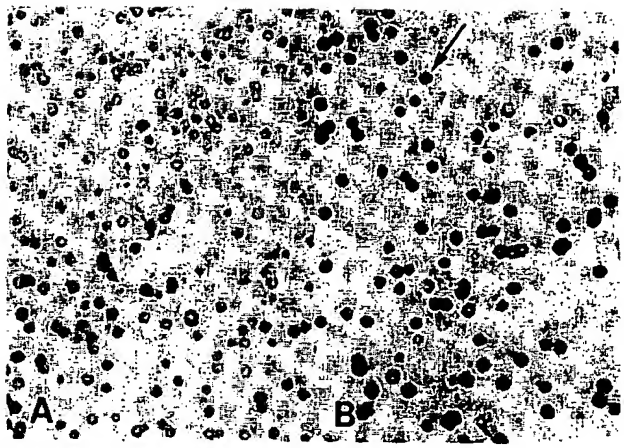

Figure 1. Detection of C8-2 antibody in chimpanzee sera by immunoplaque assay. Phage containing the C8-2 and negative control phage were mixed (1:1), and plated on agar. Replicate filters were incubated with chimpanzee serum obtained in week 0 (A) or 20 (B) during the chronic phase after inoculation of the F-strain of NANBH agent as the first antibody. Approximately half the plaques in B give a positive reaction, as shown by an arrow.

Table 1. Incidences of antibodies against C8-2 antigen in experimentally infected chimpanzees*

Diagnosis	No. positive/No. examined	(%)
NANBH, convalescent	0/7	(0)
NANBH, carrier	5/6	(83.3)
Normal	0/17	(0)

*All the chimpanzees except one (No. 59) used for detecting antibodies had been inoculated with F-strain of NANBH agent. No.59 was inoculated with serum from a patient with acute epidemic NANBH in Shimizu City, and developed chronic hepatitis (14).

RESULTS

Isolation of cDNA from human plasma RNA

A library containing 1.2×10^6 plaque forming units, which was obtained from 8 liters of possibly infectious pooled plasma, was immuno-screened with sera from chimpanzees in the convalescent and chronic carrier phases of non-A, non-B hepatitis (NANBH). One of 3 positive cDNA obtained, clone C8-2, was specifically associated with the diagnosis of NANBH: on immunoplaque assay

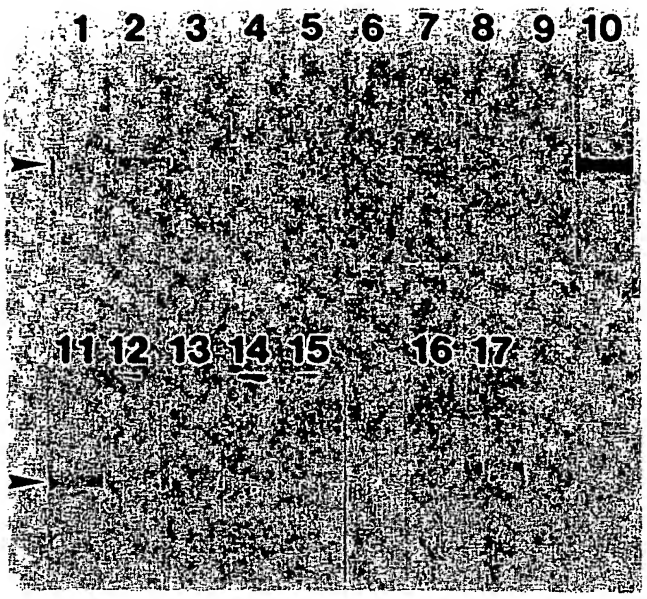

Figure 2. Detection of C8-2 antibody by Western blot analysis in human sera from patients with chronic NANBH (lanes 1–15), chronically infected chimpanzee serum, as a positive control (lane 17) and uninfected chimpanzee serum, as a negative control (lane 16). C8-2/lacZ fusion protein was separated as a band of approximately 160 kd by SDS-PAGE (arrow heads). Lanes 2, 7, 10, 11, and 13 gave a positive reaction.

as shown in Fig. 1, antibodies to C8-2 were frequently detected in the sera of chimpanzees in the carrier phase of NANBH (83.3%), but not in convalescent sera or in sera of normal chimpanzee (Table 1). In serial examinations of 4 chronic carrier chimpanzees, the antibody to C8-2 antigen was found to appear 20 weeks after inoculation in 3 chimpanzees, and 102 weeks after inoculation in one (data not shown). These results show that this antibody appears in the chronic phase, but not the acute phase of infection. The antibody did not appear in a chimpanzee infected with hepatitis B virus (HBV)(data not shown).

Close association of appearance of C8-2 antibody with NANBH in human serum

The specific incidence of C8-2 antibody in serum was also confirmed in the sera of human patients with a well-defined clinical course. As shown in Table 2, by immuno-plaque assay, the C8-2 antibody was detected in 40.0% of the sera of chronic cases and 10.0% of the sera of acute stage cases of NANBH, but was not detected in sera of patients with acute hepatitis A or B. The serum from one patient with chronic hepatitis B was positive, suggesting infection with both NANBH virus and HBV. The C8-2 antibody appeared with low frequency (1.2%) in sera of normal blood donors whose ALT values were less than 30 mU/ml. As shown in Fig. 2, Western blot analysis revealed that sera that were positive for C8-2 antibody reacted specifically with a band of 160 kd of C8-2/lacZ fusion protein. Western blotting gave almost the same results as immunoplaque assay (see Table 1), but 2 serum samples from chronic NANBH patients gave a positive reaction on Western blotting but a negative one on immunoplaque assay, and 2 gave a negative reaction on Western blotting but a positive one on immuno-plaque assay. One sample from a patient with chronic hepatitis B also gave a positive reaction only on Western blotting analysis (Table 2).

Table 2. Specific appearance of antibodies against C8-2 in human blood samples detected by immunoplaque or Western blot assay.

Diagnosis	No.positive/No.examined (%) Immunoplaque assay	Western blot assay
NANBH, acute	4/40 (10.0)	4/40 (10.0)
NANBH, chronic	12/30 (40.0)	12/30 (40.0)
HBV, acute	0/20 (0)	0/20 (0)
HBV, chronic	1/20 (5.0)	2/20 (10.0)
HAV, acute	0/20 (0)	0/20 (0)
Other liver diseases*	0/15 (0)	0/15 (0)
Normal	1/83 (1.2)	ND**

*The patients consisted of 5 with alcoholic liver injury, 5 with drug-induced liver injury, and 5 with lupoid hepatitis.
**Not done.

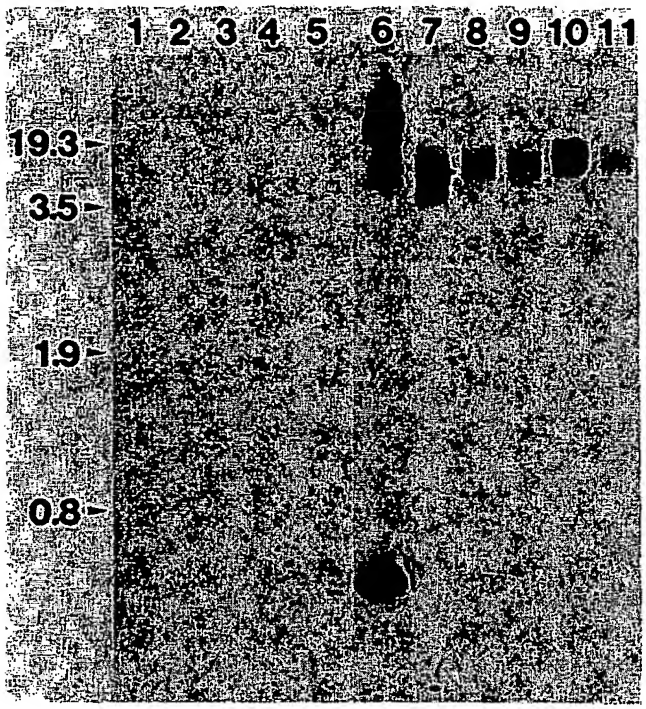

Figure 3. Southern blot analysis of liver DNA from 3 chimpanzees (lanes 1–3, 7–9), leukocyte DNA from 2 humans (lanes 4–5, 10–11), and lambda C8-2 phage DNA (lane 6). Samples of 30 μg of genomic DNA or 2 μg of phage DNA were digested with *EcoRI*, and then separated by electrophoresis in agarose gel, transferred to a nitrocellulose filter, and hybridized with C8-2 (lanes 1–6) or beta-actin (lanes 7–11) probe.

As further controls for other liver diseases, we tested for this antibody in sera of patients with clear diagnoses of alcoholic liver injury, lupoid hepatitis, and drug-induced liver injury (5 samples of each). No antibody was detected in any of these 15 samples by either immunoplaque or Western blot assay, indicating that the presence of C8-2 antibody was restricted to NANBH.

Characterization and sequencing of C8-2 clone

To prove that this cDNA clone was not of endogenous origin, we excised the DNA from the plasmid subclone, C8-2-5, and used it as a probe in Southern blot analysis of chimpanzee and human genomic DNA (Fig. 3). The control beta-actin gene probe hybridized with all samples of *EcoRI* digests of genomic DNA,

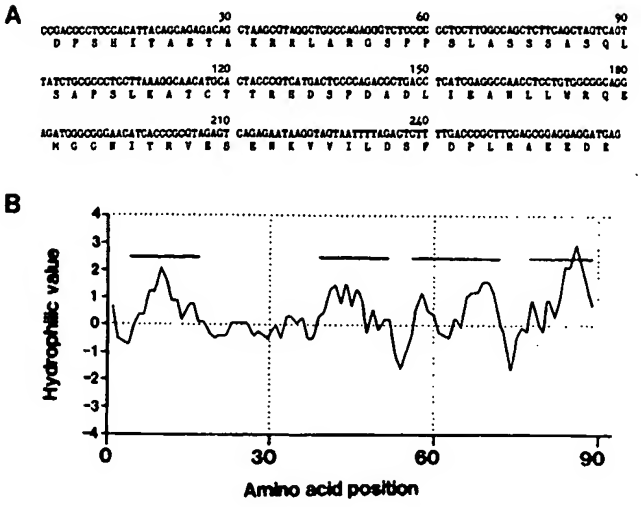

Figure 4. DNA sequence and deduced amino acid sequence of the 0.28 kb fragment derived from C8-2-5 (A) and hydropathic analysis of this 89 amino acid sequence (B) according to Hoop and Woods (13). The results show that this sequence encodes a hydrophilic part of the protein with 4 main hydrophilic regions (bar).

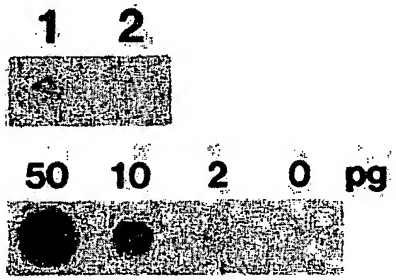

Figure 5. Detection of RNA in the human serum-derived viral fraction with C8-2-5 probe. Samples of total nucleic acid isolated from 50 ml of pooled plasma from blood donors with elevated ALT was spotted on a nitrocellulose filter after no treatment (lane 1) or treatment with RNase (lane 2). Standard hybridization of C8-2 probe to lambda gt11 phage DNA containing 50, 10, 2, and 0 pg of C8-2 DNA was shown in lower.

but the C8-2 insert DNA probe did not hybridize with any chimpanzee or human DNA. These results demonstrate that this C8-2 clone was not derived from host genomic DNA, but was of exogenous origin.

We then sequenced 0.28kb of an exogenous fragment in plasmid C8-2-5. Figure 4A shows the nucleotide sequence of 269 base pairs and its deduced amino acid sequence. This region had a high G/C content (58.7%), and 4 main hydrophilic regions, as shown by hydropathic analysis according to Hopp and Woods (13) (Fig. 4B). Homology search using DNASIS software (Hitachi, Tokyo) showed no significant homology (less than 50%) with any nucleotide sequence of primates or viruses filed in GenBank (June, 1989) or EMBL (May, 1989).

Detection of specific RNA in human plasma

Purified nucleic acid derived from 50 ml of pooled plasma from human blood donors whose ALT levels were more than 100 mU/ml were spotted on a nitrocellulose paper, and hybridized with C8-2 specific probe. A signal was detected with total nucleic

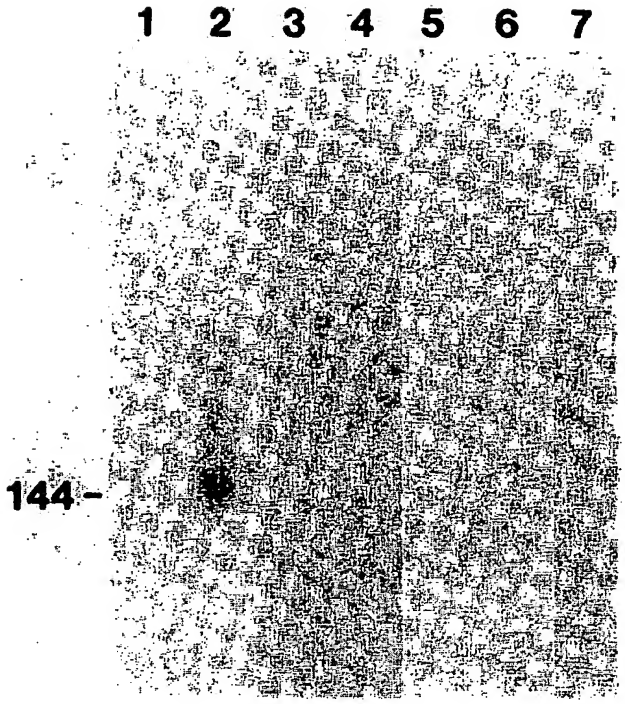

Figure 6. Southern blot analysis of products amplified by the polymerase chain reaction. Total nucleic acid from pooled plasma of chimpanzees during the chronic carrier phase (lane 1), pooled human plasma with elevated ALT (lane 2), and human plasma of two healthy individuals (lanes 3 and 4), were reverse-transcribed, amplified, and analyzed by Southern blotting as described in the Materials and Methods. Total RNA purified from the livers of two chimpanzee in the acute phase (lanes 5 and 6) was specifically amplified in the same manner. The samples subjected to electrophoresis were obtained from 15 μl of plasma, or 200 ng of liver RNA.

acid from plasma with an elevated ALT level (Fig. 5). This signal disappeared after treatment with RNase, indicating that it was derived from RNA in the serum.

The concentration of the C8-2 sequence in serum RNA of patients was estimated to be as low as 40 fg/ml from the results of dot hybridization shown in Fig. 5. Therefore, for its detection we developed a gene amplification system by the polymerase chain reaction (PCR) with specific primers as described in the Materials and Methods. As shown in Fig. 6, a specific signal was observed by Southern blot analysis in pooled human plasma with high ALT values, but not in 2 samples of normal plasma. The signal was also not detected in 2 samples of liver from chimpanzees in the acute phase, or a sample of serum from chimpanzee in the chronic phase of NANBH after infection with the F-strain of agent. Thus the C8-2 sequence was detected in the sera of Japanese patients, but interestingly, nor in sera from chimpanzees infected with the F-strain of NANBH agent.

DISCUSSION

In the present work, by screening a lambda gt11 expression library derived from RNA of human sera suspected of being infectious, we isolated a cDNA clone that was closely associated with NANBH. This method may be better than others for screening for a gene derived from a rare infectious agent such as NANBH virus because specific antibody against the agent

might appear in large amount in the serum, even if the amount of agent is very low. Furthermore, with this method it is possible to speculate on the function of the protein encoded by the isolated cDNA clone, because the recombinant protein expressed in *E. coli* must contain one or more epitopes that are specifically recognized by antibodies in the patient's serum.

Although our results showed that the C8-2 clone was closely associated with NANBH, we cannot conclude from this finding only that this clone was derived from this hepatitis agent. But from several other findings we suggested that this clone was in fact derived from the agent of hepatitis. First antibody against C8-2 protein was detected in 40% of the sera of patients with chronic NANBH, but in only one of the patients with other forms of viral hepatitis examined and in none with alcohol liver injury, lupoid hepatitis, or drug-induced liver injury (Table 2). Second the nucleotide sequence showed no homology with those of known primate and viral nucleotides, or with human or chimpanzee genomic DNA as judged by Southern blot analysis (Fig. 3). Third amplified product blotting of nucleic acids from pooled plasma of blood donors with elevated ALT activity hybridized with the C8-2 probe, whereas nucleic acid from the plasma of normal donors did not (Fig. 6), and this sequence in the plasma was an RNA-derivative (Fig. 5). Thus the RNA sequence corresponding to that of C8-2 was present only in human plasma with elevated ALT activity.

The recent findings by Choo and collaborators (4, 5) on the specific appearance of antibody to C100-3 recombinant antigen in NANBH patients are relevant to ours. The main difference between these two studies is that they found that more than 70% of patients with chronic NANBH were positive for C100-3, whereas we found that only 40% were positive for C8-2. This difference might be partly due to low sensitivity of immunoplaque assay for detecting antibody. But it was probably mainly due to a difference in the appearance of these two antibodies against distinct epitopes. One reason for this conclusion is that the C8-2 antibody was present in a high percentage (83.3%) of chronic carrier chimpanzees, whereas a lower percentage would be expected if the difference in findings were mainly due to a difference between the sensitivities of these two assays. Furthermore, in a preliminary study we found that C100-3 and C8-2 antibodies were detected at almost the same ratio (18.2% and 16.7%, respectively), in the sera from 148 voluntary blood donors whose ALT levels were more than 100 mU/ml, suggesting similar sensitivities of these two assays. Interestingly, 4 cases gave positive reactions for C8-2 but not for C100-3 antibodies, and 7 cases gave positive reactions for C100-3 but not for C8-2 antibodies. These findings suggest that our clone and clone 81 of Choo *et al.* are derived from different viruses or different regions of a common viral gene.

In recent reports Arima *et al.* (15, 16) described two cDNA clones isolated from the plasma of human patients, one associated with the acute and chronic phases of NANBH, and the other with only the chronic phase. The nucleotide sequence of C8-2 was different from those of their two clones, indicating that these sequences are also derived from a different virus or different regions of a common viral gene.

As shown in Fig. 5, little, if any of the specific RNA corresponding to the C8-2 sequence was present in infectious plasma. Moreover, the C8-2 probe did not hybridize with total liver RNA from the two chimpanzees in the acute phase of NANBH (data not shown). Therefore, it was necessary to develop a system for amplifying the complementary sequence in patient's sera. Interestingly using a PCR system we found that this amplification based on the Japanese cDNA clone, C8-2, did not function in detection of the F-strain genome of the NANBH virus (Fig. 6). This suggests that the C8-2 gene and F-strain of virus have somewhat different base sequences but share the same epitope encoded in the C8-2 region. This suggestion is supported by the the fact that a very high proportion (83.3%) of chimpanzees in the chronic phase after infection with the F-strain gave positive reactions for antibodies to C8-2 antigen.

In this study we isolated a cDNA clone derived from infectious human plasma. This clone was shown to be closely associated with chronic NANBH, and might encode a different viral epitope from that described by Choo *et al.* (4). This finding should provide fundamental information on the whole gene of this virus and the functions of individual viral antigens.

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